## Current events

# Oral bacteria in Miocene *Sivapithecus*

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In human, as well as other animals studied to date, the interrelationship between host and associated resident microbiota plays a critical role in health and disease. The complexity of such interactions is most evident in the human mouth, which can play host to as many as 500 different adherent microbial species (Moore & Moore, 1994). Whereas in some cases the resident oral microbiota play a protective role (e.g., in preventing colonization and

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proliferation of candida species), oral bacteria are also involved in two of the most prevalent current diseases of mankind, i.e., dental caries and periodontal disease (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Moore & Moore, 1994; Nisengard & Newman, 1994), and may even be linked to coronary heart disease (DeStefano *et al.*, 1993). Both these diseases are considered to be associated with colonization of the tooth surface by certain strains of particular species. Dental caries are thought to be associated with mutans streptococci, although *Actinomyces* and lactobacilli have also been considered to play a role (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Nisengard & Newman, 1994). Periodontal diseases may involve a wide variety of proteolytic Gram-negative species, including *Fusobacterium*, *Porphyromonas gingivalis*, and spirochetes (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Moore & Moore, 1994; Nisengard & Newman, 1994). Although evidence for both these diseases have been found in skulls of early Pleistocene hominids (Clement, 1958; Ripamonti, 1988), little is known of the ancient microbial makeup of hominoid/hominid dental plaque.

Calculus is a mineralized plaque that adheres to the tooth surface and is covered by an unmineralized bacterial layer (Gibbons & Van Houte, 1973; Caton & Quinones, 1991; White, 1991). Within the mineralized matrix bacteria are the main component with some epithelial cells, blood cells, and remnant food debris (Friskopp & Hammarstorm, 1980; Dobney & Brothwell, 1986; Linossier *et al.*, 1988; Hershkovitz *et al.*, 1997). Owing to its mineral content, dental calculus survives well in fossils (Hershkovitz *et al.*, 1997; Vandermeersch *et al.*, 1994).

In the present study, we have examined the dental calculus obtained from the fossil ape *Sivapithecus*. Specimens of this primate are known from middle to late Miocene sediments of South Asia (ca. 12·5–8·5 million years bp) (Pilbeam *et al.*, 1977). *Sivapithecus*, is generally attributed to the extant orang-utan lineage, based on a number of presumed shared derived cranial features (Andrews, 1992).

The studied specimens were collected from exposures of the Siwalik Sequence on the Potwar Plateau, Northwest Pakistan (Pilbeam *et al.*, 1982). Dental calculus was present in two of seven *Sivapithecus* specimens (some represented solely by isolated teeth): GSP (Geological Survey of Pakistan) 15000 and GSP 49567. GSP 15000 consists of a partial skull and complete mandible, recovered *in situ* from overbank sediments paleomagnetically dated to ca. 9·3 million years bp. GSP 49567 is a partial mandible recovered as a surface find from similar sediments, dated to ca. 8·7 million years bp.

Chips of *Sivapithecus* supragingival calculus were pried off the tooth surface of the lower second left molar of GSP 15000 and second molar of GSP 49567, using dental scaler (in this manner the calculus was entirely removed from tooth surface). The calculus samples were examined by scanning electron microscopy in two successive stages (Figure 1). Preliminary classification of biologic forms (e.g., bacteria) was done on the basis of shape and size (Boyde & Williams, 1971; Holt, 1984). To ease identification, stereoscopic images, obtained by tilting the stage 8° between two exposures of the same location, were obtained. The preserved microbiota in the two samples of *Sivapithecus* dental calculi were compared with calculi microbiota in Pleistocene and modern humans and in apes. Comparative samples included dental calculus (sub- and supragingival) from the second and third upper and lower molars of 15 free-ranging apes (five *Pan troglodytes*, five *Gorilla gorilla* and five *Pongo pygmaeus*) and 15 modern humans (Hamann-Todd collection, The Cleveland Museum of Natural History), one Neandertal, Spy I (Laboratoire d'Anthropologie et de Genetique humaine, Universite Libre de Bruxelles), plus 15 prehistoric (natufian) hunter–gathers (ca. 10,500–8300 B.C.) and 15 early neolithic farmers (ca. 8300–6500 B.C.) (Department of Anatomy collection, Tel Aviv University).

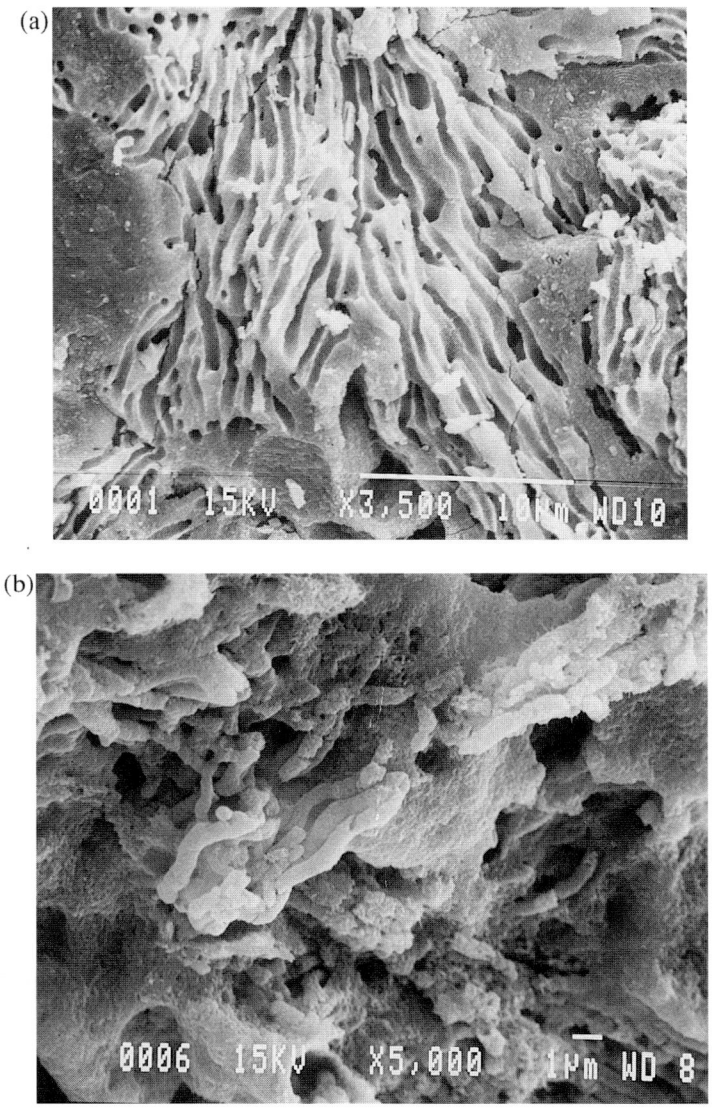


Figure 1. Scanning electron micrographs of GSP 15000 calculus. (a) Stage 1: Calculus samples were sectioned, cleaned, and affixed to an aluminum specimen mount with double sticky carbon tape so that the exposed split surface of the calculus could be examined. Specimens were coated with 20 nm palladium/gold in an RMC-Eiko IB-3 ion coater, and examined in a JOEL 840-A scanning electron microscope. Impressions of filamentous micro-organisms, appearing as shallow grooves, holes and cross-cut channels, were observed. (b) Stage 2: A few samples were removed from specimen mounts, immersed in 1N HCL for 2 min, rinsed thoroughly in distilled water, and dried in a 65°C oven for 30 min. Digested samples were gently collected with double sticky carbon tape and affixed with the calculus to an aluminum specimen mount. The samples were coated again and reexamined. Fossilized bacteria are clearly evident.

The fractured surfaces of the supragingival calculus of GSP 15000 had a smooth, solid appearance (Figure 1). Scanning electron microscopy revealed the microbial composition of the fossilized calculus to be remarkably homogeneous, with similar looking filamentous morphotypes comprising approximately 99% of the microbial fossils observed (Figure 1).

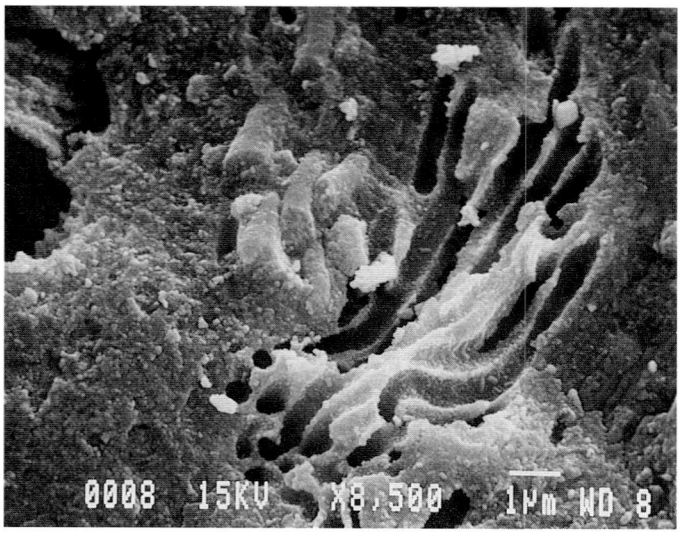


Figure 2. Scanning electron micrographs of GSP 49567 calculus. Shallow grooves are indistinguishable in appearance from those seen in Figure 1(a). Notice the fossilized bacteria adjacent to the grooves.

The filamentous bacteria, $0{\cdot}5 \pm 0{\cdot}1$ μm in diameter, appeared to be aligned and tightly packed in the center of the calculus (i.e., half way from the tooth surface to the plaque exterior), and extended outwards, in an increasingly random fashion, from 10–50 μm from the fractured matrix (being partially embedded within the matrix). Straight or slightly curved short bacilli (0·3–0·9 μm in diameter and 1·5–5·0 μm in length) and coccal forms (ca. 0·4–0·9 × 0·4–0·9 μm) were rarely observed. The matrix of GSP 49567 contained filamentous structures identical to those of GSP 15000 (Figure 2). Several isolated 1·3 μm diameter filaments were also present in this specimen. The filaments observed in the *Sivapithecus* samples are similar in size and shape to *Actinomyces* and *Nocardia*, current common residents of dental plaque. The 0·5 μm cross-section is too small for fungal filaments, and septae characteristic of algal forms were not found.

Whereas dental plaque samples from contemporary humans and apes also contain filamentous bacteria, especially in mature plaque, they appear to be much more heterogeneous in appearance and distribution (Figure 3), and share the calculus matrix with a variety of cocci and bacilli. In *Sivapithecus*, in contrast, bacilli and coccal forms were rarely observed (less than 1%).

As bacteria are an inherent component of dental calculus (Newman & Britton, 1974; Lustmann *et al.*, 1976; Listgarten, 1986; Carrassi *et al.*, 1988), observation of a filamentous form in essentially pure "culture" would seem to exclude the possibility of simple post-mortem contamination, for example by soil organisms such as *Streptomyces*. Absence of external surface invasion of the calculus by filamentous forms mitigates against the possibility that the observed structures result from post-mortem contamination. Furthermore, the cells appeared to proliferate outwards from the tooth surface, as generally observed in dental calculus (Friskopp & Hammarstorm, 1980; Nisengard & Newman, 1994), whereas a contamination would show the opposite orientation. The observed forms respect physiologic constraints (e.g., oriented predominantly perpendicular to the tooth surface) (Friskopp & Hammarstorm, 1980; Nisengard & Newman, 1994), in contrast to contaminants which permeate entire structures.

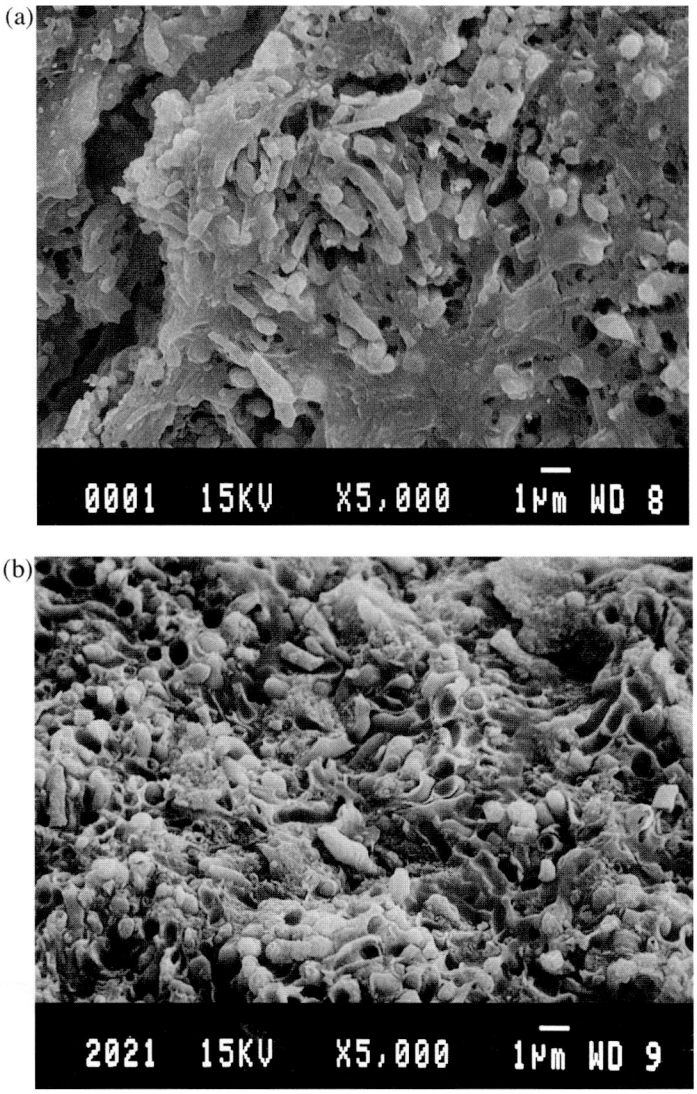


Figure 3. Bacterial colonies in chimpanzee (*Pan troglodytes*) calculus (Left B-1434; Right B-1806, Hamann-Todd collection). Note the heterogeneous nature of the bacterial types.

To our knowledge, this is the first study to observe dental micro-organisms in fossils more than 60,000 years old. In a recent study on Neandertal teeth, Vandermeersch and coworkers (Vandermeersch *et al.*, 1994) observed that the composition of bacteria in Neandertal calculus is very similar to that of modern humans. Yet, our data raise the possibility that in early hominids and hominoids, the oral microbiota was much less complex than in current humans or apes. Similarly, the increases in prevalence of both dental caries and periodontal disease observed during the last few thousand years might possibly be related to relatively sudden changes from relatively homogeneous to extremely heterogeneous dental plaque (Hershkovitz *et al.*, 1997). Whether such postulated changes may have derived from differences

in diet, social behavior, immunological considerations, or association with domesticated animals is a matter of speculation.

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# Hydrophobic Interactions and Inhibition of Microbial Adhesion

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*Abstract*

Over the past few decades, it has become clear that hydrophobic interactions are an important factor in microbial adhesion to a wide variety of surfaces. This includes not only non-wettable surfaces, but a variety of substrata in the environment and host. Various cell surface components that promote adhesion *via* hydrophobic interactions (»hydrophobins«) or interfere (»hydrophilins«) have been identified. The ability of soluble amphipathic agents to interfere with adhesion has been tested using a variety of components and test systems. Another approach which we have used is the so-called BAWA (»beat adhesion with adhesion«) in which adhering bacteria can be desorbed from a surface by preferential adhesion onto the oil : water interface. We have successfully used oil droplets to desorb bacteria from oral surfaces, and since 1992, a proprietary twophase mouthwash has been commercially available in Israel, based on this scientific principle.

## 1. Introduction

Cell surface hydrophobicity has become of increasing interest during the last decade. Hydrophobic interactions play an important role in different aspects of microbial ecology. Examples include adhesion to solid surfaces, partitioning at liquid:liquid and liquid:air interfaces, nutrient scavenging, gliding and spreading, and interactions with phagocytic cells (Rosenberg and Kjelleberg 1986, Rosenberg and Doyle 1990). Medically-related microbial adhesion influenced by hydrophobic interactions include adhesion to teeth, fatty tissues, host insect cuticle, catheters, intrauterine contraceptive devices, bioprostheses, contact lenses, and other biomaterials, as well as a variety of epithelial and phagocytic cell types (for a review see Rosenberg and Doyle 1990). During the last decade, many methods were developed for assessment of cell surface hydrophobicity, each with its advantages and disadvantages (for a recent technique, see Straver and Kijne 1996). In 1980, we proposed a rapid method for assessing microbial cell surface hydrophobicity, based on adhesion to oil droplets. In this assay, washed cell suspensions are vortexed with the test liquid hydrocarbon (mainly n-hexadecane), and the degree of adhesion of cells to the oil droplets is measured spectrophotometrically following phase separation (Rosenberg et al. 1980). Although other tests for cell surface hydrophobicity have been developed, the simplicity of the so-called MATH (microbial adhesion to hydrocarbons) made it a popular assay (Bar-Ness Greenstein et al. 1995, Lin et al. 1995), but their discussion is beyond the scope of this chapter; for reviews see Rosenberg and Doyle 1990, Rosenberg et al. 1991a, van der Mei et al. 1991). The MATH test subsequently led us to seek agents which can prevent or reverse adhesion by interfering with hydrophobic interactions.

Conversely, we found ways to promote adhesion to oil droplet, leading us to develop a novel two:phase microbe-desorbing mouthrinse, based on the BAWA (»beat adhesion with adhesion«) approach.

## 2. Adhesion and Microbial Cell Surface Hydrophobicity

Generally, the attachment of bacteria to surfaces depends on several factors, including the properties of the substratum, the liquid phase, and the bacteria themselves (PRINGLE and FLETCHER 1986).

Biofilm formation occurs on virtually any surface in any environment in which bacteria are present. The nature of the growth environment has a profound influence on the regulatory mechanisms determining surface properties of bacteria. The gram-negative cell envelope shows remarkable flexibility in both structure and composition in response to changes in the surrounding environment (VAN LOOSDRECHT et al. 1987). ALLISSON et al. (1990) studied the possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. They found no significant differences in surface hydrophobicity for cells grown either planktonically or as biofilms. Both populations became less hydrophobic as the growth rate was increased. Cells increased in surface hydrophobicity upon the onset of the stationary phase. These results indicate that changes in surface hydrophobicity may be associated with the division cycle and that surface hydrophobicity immediately after division is low. Hence, bacteria dislodged from these biofilms (due to low surface hydrophobicity) provided an inoculum of new cells capable of replication and colonization of new surfaces. The opposite trends of surface hydrophobicity and growth rate may imply that the changes in surface hydrophobicity reflect alterations of the cell surface with respect to surface appendages or proteins associated with adhesion. Such adhesive structures are minimized during and immediately after division, leading to daughter cell separation and dispersal (ALLISON et al. 1990).

The above trend of surface hydrophobicity and growth rate found in *E. coli* was not valid in the case of *Pseudomonas* sp. In *Pseudomonas aeruginosa* hydrophobicity increased with growth rate (VAN LOOSDRECHT et al. 1987). In *Pseudomonas aeruginosa* it was found that bacterial cell surface hydrophobicity is the major parameter influencing the kinetics of adhesion to stainless steel. The bacterial cell surface charge seemed of minor importance in this process, as seen in fifteen different isolates of this strain (VANHAECKE et al. 1990).

There are many examples of changes in surface hydrophobicity during procaryotic development, in gram-positive as well gram-negative bacteria (ROSENBERG and SAR 1990). One example is the change in cell surface hydrophobicity during the formation of spores. In *Myxococcus xanthus*, a gram-negative bacterium, sporulation was accompanied by a dramatic decrease in surface hydrophobicity (KUPFER and ZUSMAN 1984). The opposite trend was found in the gram-positive bacterium *Bacillus subtilis* and several other *Bacillus* sp. DOYLE and coworkers have reported that all *Bacillus* spores tested were more hydrophobic as compared with the corresponding vegetative cells (DOYLE et al. 1984). Another example of the influence of growth phase and growth conditions on bacterial adhesion to surfaces, is demonstrated in several gram-negative bacteria i.e., *Enterobacter cloacae*, *Pseudomonas fluorescens* and *Flexibacter* sp. The cell surface hydrophobicity of these strains varied with growth phase and with the

growth medium used. Therefore, the adhesion ability of these bacteria to polystyrene was affected as well (McEldowney and Fletcher 1986).

Growth temperature may also affect adhesion ability of bacteria to different surfaces. In *Serratia marcescens*, all strains tested were adherent following growth at 30 °C whereas none retained adherence properties following growth at 38 °C (Rosenberg et al. 1986).

The significance of the substratum properties in facilitating microbial adhesion has been studied intensively. Attachment of bacteria to surfaces has been shown to be influenced by factors such as surface charge, surface-free energy, and the presence of adsorbed macromolecules. Whenever a solid surface is immersed into an aqueous solution, solutes are quickly adsorbed onto the surface. Macromolecules are generally irreversibly adsorbed and have a subsequent effect on bacterial attachment to the surface. The hydration of a surface as well as the adsorption of macromolecules can reduce bacterial attachment. However, additional factors, relating to the chemical composition of the substratum and the stabilization of the suspended bacteria, can effect the adhesion interaction and the resulted number of attached bacteria (Pringle and Fletcher 1986).

The bacterial cell surface plays an important role in adhesion to oral surfaces, including both hard and soft tissues (van der Mei et al. 1990). As in the case of other surfaces, adhesion of oral microorganisms to mouth surfaces is governed by a complicated interplay between the properties of the adsorbed proteins on these surfaces and the characters of the surfaces themselves (Pratt-Terpstra and Busscher 1991).

In the case of bacterial adhesion to teeth, bacterial repopulation is promoted by intrageneric coadhesion (coaggregation among oral species, e.g. pairs of streptococci and actinomyces; Kolenbrander et al. 1990). Although the role of bacterial hydrophobicity is not inherently obvious in the coaggregation process, many adherent oral bacteria exhibit hydrophobic cell surfaces (Weiss et al. 1982). Also, most freshly oral isolates as well as many laboratory oral species are able to adhere to oil droplets after brief mixing periods (Ofek et al. 1983, Shah et al. 1989). Rosenberg et al. used *Porphyromonas* (*Bacteroides*) *gingivalis*-coated hexadecane droplets to study the coadhesion of *Actinomyces viscosus* with *P. gingivalis*. They suggested that initial attachment of individual *A. viscosus* cells to *P. gingivalis* cells may occur through complementary (but not lectin mediated) stereochemical associations. Subsequent attachment of additional *A. viscosus* is greatly promoted by lateral cell-cell interactions with other bound *A. viscosus* cells, in addition to attachment to the *P. gingivalis* cells. Such »like-like« associations may depend more on hydrophobic interactions. This model can account for the inhibition of coadhesion by defatted BSA, which is known to be a potent inhibitor of microbial adhesion to hydrophobic surfaces (Rosenberg et al. 1991 b).

The issue of bacterial attachment to surfaces has implications on the biocompatibility of biomaterials. Most of the research on this subject was performed on gram-positive bacteria (i.e. streptococci and staphylococci), whereas gram-negative bacteria are probable candidates for causing the most severe problems of undesirable adhesion. Several nonionic surfactants were found to prevent adhesion of a *Pseudomonas* strain and a *Serratia marcescens* strain to hydrophobic surfaces but not to hydrophilic ones (Humphries et al. 1987). This antiadhesive effect could have important applications in many areas where bacterial fouling is a major problem (Humphries et al. 1987).

## 3. Hydrophobins and Hydrophilins

The term »hydrophobin« was originally coined by ROSENBERG and KJELLEBERG (1986) to denote hydrophobic surface components that promote cell surface hydrophobicity and adhesion. Conversely, cell surface components that reduced hydrophobic surface properties of microbial cells were termed »hydrophilins«. In recent years use of the term hydrophobin has been extended by WESSELS and coworkers to include hydrophobic surface components of fungal cells that may or may not be involved in adhesion processes (WESSELS et al. 1991 a, b). A summary of the work done in other laboratories on hydrophobins and hydrophilins of several microbial species is presented below.

### *3.1 Acinetobacter calcoaceticus*

*Acinetobacter calcoaceticus* species, although widespread in the environment, can also inhabit the human body and are sometimes associated with nosocomial infections. Our studies concentrated on strain RAG-1, a bacterium which degrades oil and has been exploited commercially. Stationary phase cells were found to exhibit a high affinity for the oil:water interface. Mutant MR-481, selected for its inability to adhere to oil droplets, was devoid of thin fimbriae present on wild type cells. Revertants that regained partial adhesion characteristics were found to possess copious thin fimbriae (ROSENBERG et al. 1982). These data suggested that the thin fimbriae function as hydrophobins of RAG-1 cells. However, another RAG-1 mutant was found to be able to adhere, albeit with somewhat lower affinity, at the oil:water interface, even though it lacked thin fimbriae. Thus, other RAG-1 hydrophobin(s) remain to be elucidated.

Emulsan is a capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 which is sloughed off (together with some bound protein) into the growth medium, when cells reach stationary phase. Emulsan exhibits amphipathic properties and is a potent emulsifier of oil droplets. When free in solution, emulsan acted as a potent inhibitor of microbial adhesion (see further). We originally hypothesized that it might act on the cell surface to promote adhesion. However, the opposite turned out to be the case. Cells, after having shed their emulsan, became increasingly hydrophobic. Similarly, the rhamnose-rich capsule of another strain, *Acinetobacter calcoaceticus* BD 4, although completely different in composition, also prevented adhesion to hexadecane while on the cell surface. Concomitant with enzymatic degradation of the capsule on the cells themselves, adhesion to oil droplets increased from zero to almost 100% (E. ROSENBERG et al. 1983 a, b).

### *3.2 Streptococcus pyogenes*

*Streptococcus pyogenes* is a common cause of throat infections, and other, sometimes life-threatening diseases. Cells of *S. pyogenes* adhere avidly to hexadecane and other oil droplets (OFEK et al. 1983). LTA and M proteins have both been considered as promoting hydrophobic surface properties of *S. pyogenes*, both individually and in association with one another. Conversely, hyaluronate capsule appears to act as a hydrophilin, and the hydrophobic-to-hydrophilic conversion of cells may be critical for successful colonization. *S. pyogenes* cells may initially be hydrophobic, to facilitate initial adhesion, but subsequently elaborate capsule to prevent phagocytosis (OFEK et al. 1983).

### 3.3 *Serratia marcescens*

*Serratia marcescens* is a widespread microbial species which can be a troublesome source of nosocomial infections, particularly in neonatal wards. It has been recognized for some time that prodigiosin, the red, oil-soluble pigment characteristic of pigmented strains, imparts hydrophobic surface characteristics, enabling cells to partition at air : water interfaces (HERMANSSON et al. 1979). Nevertheless, nonpigmented clinical strains are also capable of adhering (albeit, less avidly) to nonwettable surfaces (ROSENBERG et al. 1986). Furthermore, cells grown at temperatures above 37 °C lose pigmentation, yet remain adherent. Finally, nonpigmented mutants may retain partial hydrophobic surface characteristics (ROSENBERG 1984). BAR-NESS and ROSENBERG (1989) demonstrated that a 70-kDa surface protein, serraphobin, which is released from *S. marcescens* cell surface during growth, is itself capable of binding to oil droplets. Furthermore, a nonadherent, nonhydrophobic yet pigmented mutant, was found to lack this surface protein (BAR-NESS 1992). Transformation of *Escherichia coli* with DNA fragments from *S. marcescens* resulted in a transformant clone exhibiting an increase in hydrophobic surface properties, concomitant with appearance of a *S. marcescens* antigen (as yet unidentified) on the surface of transformant cells. Thus, additional hydrophobins may also be present on *Serratia marcescens* cells (BAR-NESS GREENSTEIN et al. 1995).

*Serratia marcescens* shows spreading growth on various surfaces (MATSUYAMA et al. 1990). The spreading growth of this strain occurs at 30 °C, a temperature which allows production of serratamolide, a known specific aminolipid wetting agent. Mutants defective in wetting agent production showed retarded spreading growth (MATSUYAMA et al. 1989). As to the influence of cell surface hydrophobicity on spreading growth of *S. marcescens*, it still remains somewhat unclear. The amphipathic wetting agent, serratamolide, appears to act as a hydrophilin, rather than as a hydrophobin. Mutants lacking this surfactant molecule exhibited higher adhesion levels than isogenic wild type cells (BAR-NESS et al. 1988).

Recent results (BAR-NESS and ROSENBERG, unpublished data), show differences in spreading behavior between three strains of *S. marcescens* differing in wetting agent activity as well as in cell surface hydrophobicity. It seems that wetting agent production, combined with cell surface hydrophobicity, plays an important role in spreading growth of these strains. The wild type strain showed the most extensive spreading pattern as well as highest spreading rate as compared to the two mutants. These data imply that cell surface hydrophobicity may account for the increased spreading growth observed in the wild type strain (ROSENBERG and BAR-NESS, unpublished data).

In general, the possible advantage of such spreading phenomenon for these bacteria may be in facilitating the ability of adherent cells to spread out over the attachment surface (MATSUYAMA et al. 1989).

### 3.4 *Candida albicans*

*Candida albicans* is an opportunistic yeast-like microorganism which can cause both superficial and systemic infections. Although hydrophobic surface properties of *C. albicans* have been reported in various studies (CALDERONE and BRAUN 1991, HAZEN et al. 1991, TRONCHIN et al. 1988, 1989), the cell surface moieties involved have not been elucidated. In order to look for potential surface hydrophobin(s) of this microorganism, a genomic library of *C. albicans* in a nonadherent strain of

*Saccharomyces cerevisiae* was constructed (BARKI et al. 1993). Adherent transformants were selected by allowing cells harboring the candidal gene library to come in direct contact with polystyrene, followed by washing to remove nonadherent cells, and overlaying a selective agar. This selection procedure yielded a transformant which exhibited enhanced adhesion to treated and untreated polystyrene. Transformant cells were also highly autoaggregative in the presence of calcium ions, as compared to *S. cerevisiae* control cells bearing the vector alone. Analysis of this transformant revealed an 4.5 kb insert originating from *C. albicans*. Curing of the plasmid resulted in loss of adhesion and autoaggregation properties. The adhesion/autoaggregation phenotype observed in transformant cells appeared due to expression of a *C. albicans* surface antigen. Rabbit antiserum, raised against transformant *S. cerevisiae* cells and adsorbed with *S. cerevisiae* bearing the vector alone, bound to the surface of transformant *S. cerevisiae* cells as well as to *C. albicans* cells (especially to the invasion-associated mycelial phase), but only marginally to the *S. cerevisiae* control. Furthermore, adsorbed antisera specifically inhibited autoaggregation of transformant cells. Western immunoblot analysis disclosed an antigen of ca. 30 kDa molecular weight, present in transformant cell surface extracts, but absent from control (BARKI et al. 1994). The extent to which this antigen acts as an adhesin/hydrophobin on *C. albicans* cells needs to be further explored.

## 4. Soluble Agents that Appear to Inhibit Adhesion via their Effect on Hydrophobic Interactions

A wide variety of amphipathic substances may inhibit adhesion to animate and inanimate surfaces (for a review see ROSENBERG and DOYLE 1990). Some examples are detergents (e.g., sodium dodecyl sulfate, Tween 80, Tween 20, Triton X-100), chaotropes (e.g., thiocyanate anion), components obtained from body fluids (e.g., bovine serum albumin [particularly defatted], normal human serum, salivary components, caseinoglycopeptides) and small organic molecules (e.g., p-nitrophenol, tetramethyl urea, propanol, fatty acids).

Amphipathic components in saliva appear to act as a barrier, preventing free microorganisms from adhering to oral surfaces. Washed microorganisms obtained directly from dental plaque were capable of adhering to buccal epithelial cells *in vitro*, but this was largely inhibited when saliva was added (ROSENBERG et al. 1989). Saliva prevented microbial adhesion to polystyrene, but when the saliva was preextracted with hexadecane, it lost its inhibitory ability (ELI et al. 1989). BABU and coworkers demonstrated inhibition of adhesion of *Streptococcus sanguis* to hexadecane by specific hydrophobic proteins of saliva (BABU et al. 1986).

One interesting aspect is the inhibition by amphipathic molecules of adhesion apparently mediated by sterospecific, lectin-like interactions. Adhesion of *E. coli* to epithelial cells, while sensitive to mannose, can also be blocked by unrelated amphipathic compounds, such as p-nitrophenol (FALKOWSKI et al. 1986). *A. viscosus* cells preferentially adhered to *P. gingivalis* cells, rather than to hexadecane, yet this ostensibly specific interaction was inhibited by defatted bovine serum albumin (ROSENBERG et al. 1991b).

The therapeutic use of antiadhesion agents in controlling microbial infection must consider that in most cases, we become aware of the intruding microorganisms after they have already adhered. In other words, for most medical purposes, antiadhesion

agents must be able to desorb already attached microorganisms, as well as to inhibit or prevent initial and subsequent adhesion. Unfortunately, prevention of initial attachment is easy, as compared with detaching microorganisms after they have already adhered, interacted with neighbouring bacteria, elaborated a capsular glycocalyx, and so on.

Relatively few studies have dealt with agents that affect microbial desorption. In one such investigation, E. ROSENBERG and coworkers were able to show that when the capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 (»emulsan«) is sloughed off into the aqueous phase, it is not only capable of inhibiting bacterial adhesion to buccal epithelial cells, but also desorbs a substantial fraction of the indigenous attached microbiota (E. ROSENBERG et al. 1983a, b).

Another consideration facing antiadhesion therapy is the observation that bacteria will eventually adhere to and foul practically every available interface. As Kevin C. MARSHALL succinctly remarked in this context: »The Bacteria Always Win.« In the following section, we describe an alternative antiadhesion strategy, i.e., the BAWA (»beat adhesion with adhesion«) approach. According to this strategy, adhering bacteria can be desorbed from one interface by preferential adhesion to a second interface. We have successfully used oil droplets to remove bound bacteria from oral surfaces in this manner (M. ROSENBERG et al. 1983a). It is important to emphasize that in such an instance, the mechanisms governing the initial adhesion are of secondary importance, as compared with the affinity of the bound cells for the disorbing interface.

## 5. Research and Development of a Bacteria-Desorbing Mouthwash

Over the past thirteen years, much of our interest in antiadhesion therapy has focused on the potential of two-phase, oil:water mixtures to remove oral bacteria and to combat bad breath. Our initial finding was that a high proportion of oral bacteria adhere to oil droplets. In 1982, NESBITT and coworkers showed that *Streptococcus sanguis* adhered to toluene droplets (NESBITT et al. 1982). In the same year, WEISS et al. reported that most oral microbial isolates retrieved from extracted teeth adhered to hexadecane. In 1983, ROSENBERG and coworkers subsequently demonstrated that washed, dispersed suspensions of microorganisms obtained directly from supragingival dental plaque adhered in high proportions to droplets of liquid hydrocarbon (ROSENBERG et al. 1983a). These initial observations led to the notion of using two-phase oil:water mouthwashes for physical desorption of oral microbiota (WEISS et al. 1985). Indeed, other studies have demonstrated that a wide variety of laboratory strains of oral microorganisms, including *Actinobacillus actinomycetemcomitans*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Streptococcus salivarius*, *Streptococcus sanguis* and certain *Streptococcus mutans* strains, adhered with high affinity to hydrocarbons (for a review, see DOYLE et al. 1990). Initial experiments showed that microorganisms which adhere to hydrocarbons also bind to other nontoxic oils (ROSENBERG et al. 1983a). Furthermore, *in vitro* experiments showed that water:oil combinations could desorb bacteria from plastic surfaces (ROSENBERG et al. 1983b). Desorption could also be demonstrated *in vivo*, based on removal of erythrosine-stained bacteria and debris from the mouth following rinsing with a two phase rinse consisting of saline and olive oil (WEISS et al. 1985). Similar combinations were also able to remove bound cells from polystyrene cuvettes (GOLDBERG and ROSENBERG 1991).

Despite these initial desorption experiments, the simple aqueous:oil combinations did not prove particularly effective in improving oral hygiene over time. Furthermore, the two immiscible oil and water phases posed technical difficulties. In 1990, GOLDBERG and coworkers found that adhesion of microorganisms and oral debris to oil was promoted by low concentrations of cetylpyridinium chloride (CPC), an amphipathic cationic agent which is commonly added to mouthwashes (GOLDBERG et al. 1990a, b). This was surprising, since amphipathic agents were generally considered to inhibit adhesion at the oil:water interface. Since CPC is an antibacterial agent, its addition had several additional advantages, including inhibition of microbial activity in the mouth, and increasing shelf life of the product by preventing microbial outgrowth. Furthermore, addition of an amphipathic agent such as CPC to oil:water mixtures results in a temporary emulsion following shaking. This in turn enabled the mixture to be supplied in a single bottle.

Prototype formulations, consisting of an aqueous phase containing CPC, and an oil phase comprising a mixture of vegetable and essential oils, were subsequently developed and tested. Such formulations maintained dramatic *in vitro* bacterial desorption properties (GOLDBERG and ROSENBERG 1991), removing over 90% of a bacterial film of *A. calcoaceticus* RAG-1 on polystyrene cuvettes, as compared to 15–50% removal for a series of commercial mouthwashes.

In 1992, the prototype two-phase oil-water mouthwash was reformulated and introduced in the Israeli market. A clinical study (KOZLOVSKY et al. 1996) was performed to compare the efficacy of the commercial version of the two-phase mouthwash with Listerine™, a mouthrinse which has been previously shown (PITTS et al. 1983, DEPAOLA et al. 1989) to be effective in reducing levels of odor-related microorganisms, as well as plaque and gingivitis.

To our knowledge, this is the first modern antiadhesion mouthrinse developed based on adsorption of microorganisms onto an alternative surface. It is however, noteworthy, that the Jewish Talmud (Bavli, Masechet Yoma, page 84), written over two thousand years ago, prescribes a mouthrinse comprising of olive oil, salt and dough water for treating oral ailments.

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